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Award Number: W81XWH-04-1-0440

TITLE: The Role of Lecithin:Retinol Acyltransferase (LRAT)-Mediated Esterification of Vitamin A in Regulating Human Breast Cancer Cell Proliferation and Differentiat

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REPORT DATE: April 2007

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-04-2007	Annual Summary	15 Mar 04 – 14 Mar 07

4. TITLE AND SUBTITLE
The Role of Lecithin:Retinol Acyltransferase (LRAT)-Mediated Esterification of Vitamin A in Regulating Human Breast Cancer Cell Proliferation and Differentiat

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-04-1-0440

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Weill Medical College of Cornell University
New York NY 10021-4870

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT: In order to understand the molecular mechanisms that mediate LRAT transcription by RA in normal human mammary epithelialcells versus breast carcinoma cells, we isolated and characterized the promoter region of the human LRAT gene and tested itsactivity in normal mammary epithelial cells versus human breast carcinoma cells. Meanwhile, in order to determine if RARγ isinvolved in the regulation of LRAT gene expression, we tested LRAT mRNA levels in Wt and RARγ -/- F9 cells, an epithelialtype of cell, which can synthesize retinyl esters. The RT-PCR analysis indicated that LRAT expression was lost in the MDA-MB-231 breast cancer cells. Surprisingly, we did not observe RA associated increase in LRAT mRNA levels in HMEC cell line inculture. By using the MatInspector V2.2 computer program, we identified two conserved half-sites separated by four nucleotides(DR-4), as well as a DR-5 RARE in the 5' flanking region of the LRAT promoter region. We isolated the 2.3-kb 5'-flankingregion of the human LRAT gene. The luciferase activity of this promoter region in MDA-MB-231 cells was one sixth of that inHMEC cells at 48 h, suggesting that this region harbors cis-elements contributing to the loss of LRAT expression in human breastcancer cells. RA treatment increases LRAT mRNA levels in F9 Wt cells at late times (72 h). There was no apparent difference inthe LRAT mRNA levels detected between the F9 Wt cells and F9 RARγ-/- cells, indicating that RARγ is not involved in thetranscriptional regulation of the human LRAT gene.

15. SUBJECT TERMS
Breast cancer, lecithin: Retinol Acyltransferase (LRAT), retinoid metabolism, Retinol, tumor differentiation, promoter, RARE, transcriptional regulation

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	12	19b. TELEPHONE NUMBER *(include area code)*

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Introduction

Mechanism of Action of Retinoids

Vitamin A (retinol) and its physiologically active metabolites (retinoids) play essential roles in physiological processes such as embryonic development, proliferation, differentiation, pattern formation, and apoptosis. Retinoids have major effects on the growth and differentiation of normal, premalignant, and malignant epithelial cells (Gudas et al. 1994). Vitamin A deficiency in animal models is associated with altered mammary gland differentiation and increased susceptibility to carcinogens (Metz et al. 2002). Retinoids have proven to be effective in suppressing breast cancer development in experimental models of carcinogenesis (Lotan 1980). In addition to breast cancer, retinoids have been shown to be effective in suppressing tumor development and/or treatment of cancers of the skin, oral cavity, lung, prostate, bladder, liver, and pancreas (Kelloff et al. 1996; Lotan 1996). The biological effects of retinoids are thought to be mainly mediated through interactions with retinoic acid receptors (RARs) or retinoid X receptors (RXRs), which function as dimeric transcription factors modulating gene expression by binding to RA-response elements (RAREs or RXREs) in the promoter regions of target genes (Chambon 1996).

Since retinoid receptor mediated transcription is governed at least in part by the relative abundance of receptor ligands, the ability of cells to generate bioactive intracellular retinoids may play a key role in controlling the growth and differentiation of cells**.** Retinol (ROL), which must be taken up by epithelial cells from the blood, is esterified within cells by the enzyme lecithin:retinol acyltransferase (LRAT) (Barry et al. 1989). LRAT plays an essential role in vitamin A metabolism and is particularly important in the processing of retinoids in the vertebrate visual cycle (Rando 2001). Although another enzyme, acetylCoA:retinol acyltransferase (ARAT), can esterify ROL, the enzyme responsible for the majority of ROL esterification in epithelial cells is LRAT (Herr et al. 1991). Retinyl esters (REs) are thought to be the storage form of ROL, and these esters can be gradually metabolized back into ROL by retinyl ester hydrolases (REH) within the epithelial cells.

Retinoid metabolism is disrupted in breast cancer cells. There are greatly reduced levels of REs in the carcinogen induced rat mammary carcinoma model (Bhat and Moudgal 1989). In contrast, normal breast epithelium contains extremely high levels of REs in this animal model. Our laboratory has demonstrated that the levels of REs in human carcinoma cell lines from the breast, oral cavity, kidney, are very low (Chen et al. 1997; Guo and Gudas 1998; Guo et al. 2001; Guo et al. 2002). In addition, we demonstrated that this low level of ROL esterification was associated with the absence of LRAT enzymatic activity, the lack of detectable LRAT protein, and abnormal *LRAT* transcripts (Guo et al. 2000). Furthermore, we demonstrated that high levels of ROL and REs were detected in normal kidney epithelial tissue, but were barely detectable in kidney tumors from patients (Guo et al. 2001). More recently, we have shown that human kidney cancers which exhibited a high level of LRAT staining by immunocytochemistry were of low malignant potential, whereas those which did not

stain for LRAT exhibited much higher malignant potential (Zhan 2003). These examples confirm the importance of LRAT in the maintenance of normal cell phenotype. When cancer cells are retinoid deficient, then retinoid signaling will be compromised. For example, the expression of RARs will be affected, given that retinoids directly regulate *RARα* and *RARβ* expression (Giguere 1994). Reduction of RAR expression will further affect retinoid signaling (Boylan et al. 1995; Isogai et al. 1997). This is particularly important as the growth inhibitory effects of retinoids are mediated largely by *RARα* and *RARβ (*van der Burg et al. *1993)*. Thus, a deficiency in esterification activity would lead to improper retinoid signaling and could contribute to carcinoma formation. In last report, we studied LRAT immunoreactivity in a variety of subtypes of human breast cancers. The data from our study indicate that the expression of LRAT decreases with a decrease in the degree of breast ductal tumor differentiation (Sheren-Manoff et al. 2006). The questions concerning how and why the expression of the LRAT gene is abrogated in breast carcinomas, is of particular important for understanding the mammary epithelial cell differentiation.

In order to understand the molecular mechanisms that mediate LRAT transcription by RA in normal mammary epithelial cells versus breast carcinoma cells, in collaboration with Dr. Kun Cai, we isolated and characterized the promoter region of the human *LRAT* gene and tested its activity in cultured normal human mammary epithelial cell line versus breast carcinoma cell lines. Meanwhile, in order to determine if RARγ is involved in the regulation of LRAT gene expression, we tested LRAT mRNA levels in the Wt and RARγ -/- F9 cells, an extraembryonic epithelial type of cell which can synthesize retinyl esters.

Body

Part 1. LRAT Expression, and Isolation and Characterization of The Human LRAT Gene Promoter

1. *LRAT* expression is reduced in human breast cancer cell lines

Normal human breast epithelial cells (HMEC) and human breast cancer cells (MDA-MB-231) were cultured, treated with 1μM all-trans retinoic acid (ATRA) or ethanol (control) for 48 and 72 h, and harvested for extraction of total RNA. 2 μg of total RNA was reverse transcribed in a total 20 μl volume reaction with 200 unit Superscript™ Reverse transcritase II (Invitrogen Life Technologies) and oligo dT (12-18) primer (Invitrogen). The cDNA thus produced was diluted to 100 μl with diethyl pyrocarbonate/water, of which 2 μl was used in a PCR with specific primers for LRAT or Cyp26A1 (a "control" gene). The PCR conditions used were as follows: 94 °C for 30 s, 58 °C for 45 s, and 72 °C for 90 s, with a final extension at 72 °C for 10 min. *Taq* polymerase was from Invitrogen (catalog number 18038-042). The PCR products were subjected to 1 % agarose gel electrophoresis. The gel images were stained with ethidium bromide and were recorded with a FluorChem 8800 system (Alpha Innotech, San Leandro, CA). The specific primers used are, for the LRAT gene, forward primer 5' GTG GAA CAA CTG CGA GCA C 3', reverse primer 5' TAG ACG CCA ATC CCA AGA CT 3'; for GAPDH,

forward primer 5' ATC CTG GGC TAC ACT GAG CA 3', reverse primer 5' TGC TGT AGC CAA ATT CGT TG 3'.

The *LRAT* mRNA was detected in HMEC cells and was not detected in MDA-MB-231 cells (Figure 1). An RA associated increase in LRAT mRNA was not observed in HMEC cells. This result indicates that normal mammary epithelial cells express *LRAT mRNA*. However, LRAT mRNA expression is abrogated in the MDA-MB-231 breast cancer cell line.

We also mesasured the *Cyp26A1* mRNA levels as a positive control. The Cyp26A1 gene has two functional RA response elements in its promoter region and is RA-inducible (Loudig et al. 2005). 1μM ATRA treatment induced *Cyp26A1* expression in both HMEC and MDA-MB-231 cells to a similar level (Figure 1). To determine if any contaminating DNA interfered with the PCR results, a no-RT control was also performed. There is no DNA contamination in the cDNA samples, as indicated by the PCR results from no-RT control (Figure 1).

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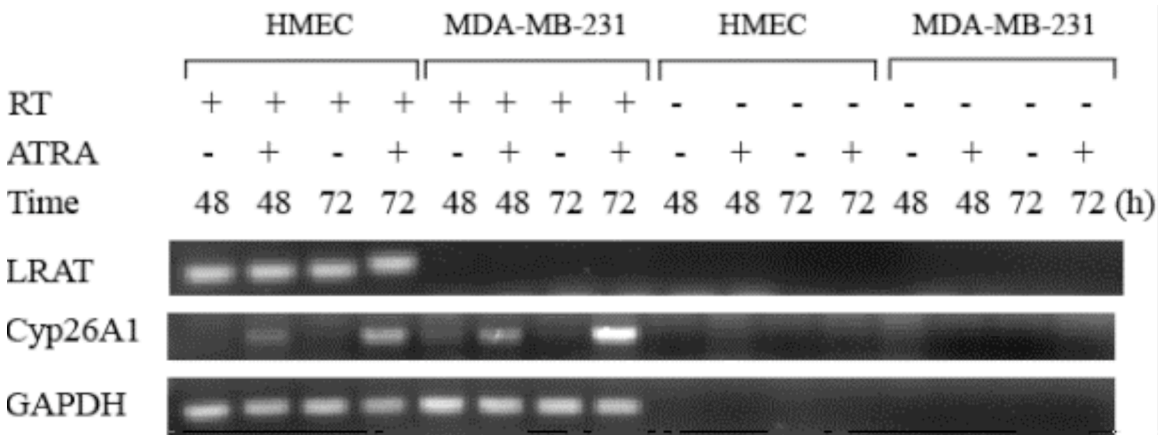

Figure 1. Expression of *LRAT* and *Cyp26A1* mRNA in HMEC and MDA-MB-231 cells. The HMEC and MDA-MB-231 cells were treated for 48 or 72 hours with ATRA (1μM) or vehicle (ethanol) as control, and total RNA was extracted using Trizol. 2 μg of total RNA was used for RT-PCR analysis with specific primers for LRAT and Cyp26A1. The level of *GAPDH* mRNA was measured as a loading control. To detect any contaminating genomic DNA, "reverse transcription" reactions were performed without reverse transcriptase (RT) and samples were subjected to PCR. The PCR products were analyzed by electrophoreses on 1% agarose gels and were stained with ethidium bromide. A representative gel is shown.

2. Putative RAREs (Retinoic Acid Response Elements) identified in the Human *LRAT* Promoter by Bioinformatics

RA treatment induces *LRAT* expression in some bladder epithelial cells and mammary epithelial cells (Andreola et al. 2000; Boorjian et al. 2004). RA might regulate *LRAT* expression through its nuclear receptors, the RARs and RXRs, which form heterodimers which bind to RAREs in the promoters of target genes (Zolfaghari and Ross 2004). RAREs generally consist of two direct repeats of the consensus sequence AGGTCA with a space between them, most commonly five nucleotides (DR-5) (Umesono et al. 1991; Mader et al. 1993; Langston et al. 1997). We searched for RAREs in the human *LRAT* 5' flanking region using the MatInspector V2.2 computer program (Quandt et al. 1995). We identified two conserved half-sites separated by four nucleotides (DR-4), as well as a DR-5 RARE in the 5'flanking region. These putative RAREs might be involved in the regulation of LRAT gene expression and need to be confirmed by further molecular functional studies (see below).

3. Construction of a LRAT promoter-luciferase reporter plasmid

To study the transcriptional regulation of *LRAT*, we constructed a human LRAT promoter-luciferase reporter plasmid. A 5'-flanking DNA region extending from -2008 through the transcriptional start site (+1) to the 5' end of the first exon at +271 of the human *LRAT* gene was amplified by PCR and inserted into the pGL 3-basic promoterless reporter plasmid to create the LRAT promoter-luciferase reporter construct, pGL-LRATP1-3 (Figure 2, top)

4. The luciferase activity of a LRAT promoter-luciferase reporter plasmid in normal breast epithelial cells and cancer cells.

The normal breast epithelial cells (HMEC) and human breast cancer cells (MDA-MB-231) were transiently transfected and then treated with 1μM ATRA, 1μM all-trans retinol, and ethanol for 48 h. Luciferase values were then recorded by a TD-20/20 luminometer (Turner Design). In HMEC cells, 1μM ATRA treatment for 48 hours caused a slight increase in the luciferase activity, whereas 1μM ROL treatment for 48 hours did not alter the luciferase activity (Figure 2). These results suggest that this 2279-bp 5'-flanking region does not harbor any *cis* element required for RA or ROL responsiveness in HMEC and MDA-MB-231 cells. Under control conditions (treatment with ethanol for 48 h), the luciferase activity in MDA-MB-231 cells was one sixth of that in HMEC cells (Figure 2), which reflects the difference in LRAT mRNA levels between the two cell lines. The difference in the promoter activity of this 2279-bp 5'-flanking region between the HMEC cells and MDA-MB-231 cells is intriguing. It suggests that this region harbors cis-elements contributing to the loss of LRAT expression in breast cancer cells.

HMEC and MDA-MB-231 cells were also transiently transfected with pGL 3-basic plasmid as a negative control and pCYP26-Luc plasmid as a positive control. When the cells were transiently transfected with the pGL 3-basic plasmid, the luciferase activity was very low (Figure 2), which indicates that the original pGL 3-basic plasmid does not contain a promoter (negative control). In the pCYP26-Luc plasmid, a fragment of the mouse CYP26 A1 promoter was inserted into a pGL 3-basic plasmid. The CYP26 promoter fragment in pCYP26-Luc plasmid contains a retinoic acid response element (RARE). In HMEC cells transiently transfected with pCYP26-Luc, 1μM ATRA treatment for 48 hours caused a 4-fold increase of the luciferase activity, while 1μM ROL

treatment for 48 hours caused a 2-fold increase in the luciferase activity (Figure 2). These results show that the ATRA and ROL we used were functional (Figure 2).

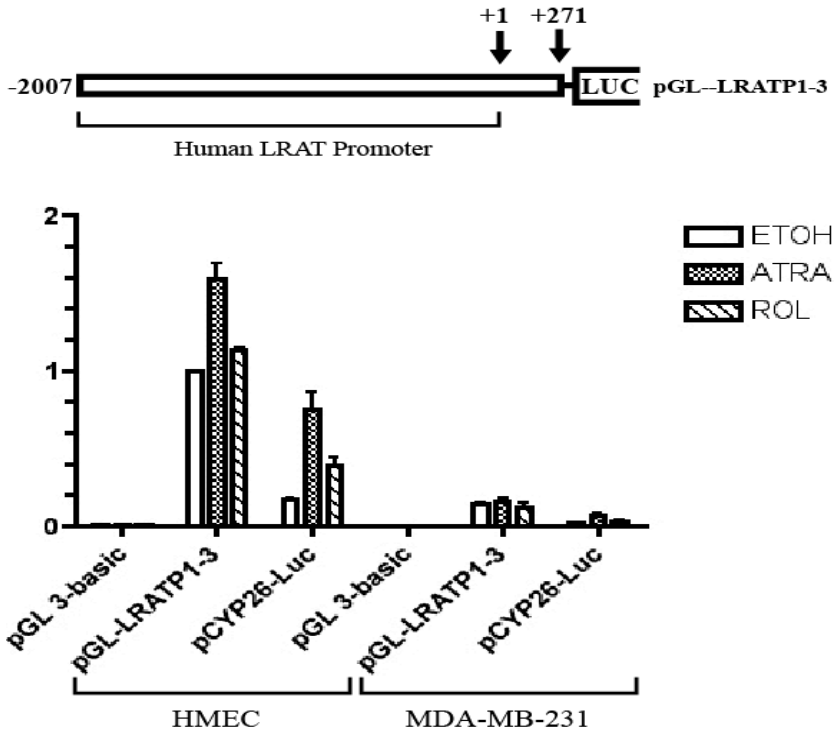

Figure 2. The luciferase activity of the 2.3 kb human LRAT promoter in human HMEC and MDA-MB-231 cells treated with ATRA or ROL. HMEC and MDA-MB-231 cells were transiently transfected with the pGL-LRATP1-3 luciferase reporter plasmid (see diagram at top), pGL 3-basic plasmid (negative control), pCYP26-Luc (positive control), and pRL-TK (for normalization of transfection efficiency) using FuGENE 6 Transfection Reagent. Then cells were treated with ethanol, ATRA, or ROL for 48 h. Luciferase activity was measured by a dual luciferase reporter assay system (Promega), and expressed relative to the value derived from HMEC cells transiently transfected with the pGL-LRATP1-3 and treated with ethanol for 48 h.

Part 2. The Role of RARγ in The Regulation of LRAT Gene Expression

Several labs have reported that RA treatment can induce LRAT gene expression and we also found a putative DR5 RARE in the 5' flanking region of LRAT gene through informatics studies. However, in our experimental system, we do not observe an RA associated increase in LRAT mRNA levels in the HMEC cells in culture. We want to test another epithelial cell line. We examined LRAT mRNA levels in F9 teratocarcinom stem

cells, which can differentiate into epithelial cells after RA treatment for 3 days (Gudas 1994) . According to our observations, there are considerable levels of retinyl esters synthesized in these epithelial cells. We found that RA treatment for 72 h dramatically induced LRAT mRNA levels. Since RAR/RXR heterdimers might directly regulate LRAT expression, and RARγ is the predominant RAR isotype expressed in the epithelial cells (Gudas 1994), we hypothesized that RARγ may also be involved in the regulation of LRAT gene expression. In order to test this hypothesis, we examined LRAT mRNA levels in the F9 Wt cells and F9 RARγ-/- cells, which were subjected to RA (1μM) treatment for 72 hours. We did not observe an apparent difference in the LRAT mRNA levels between the F9 Wt cells and F9 RARγ-/- cells, indicating that RARγ is not involved in the regulation of LRAT gene expression. This supports the concept that RARα is the predominant RAR isotype mediating RA- induced LRAT gene expression (Andreola et al. 2000).

Part 3. We generated transgenic mice in which human LRAT was ectopically expressed either in the suprabasal layer or in the basal layer of epithelial cells driven by the cytokeratin 10 promoter or the cytokeratin 14 promoter, respectively. These transgenic mice can be used to assess the functions of LRAT in breast carcinoma prevention. This portion of the project is not completed.

Key research accomplishments

1. The RT-PCR analysis indicated that *LRAT* expression was lost in the MDA-MB-231 breast cancer cells. We did not observe an RA associated increase in LRAT mRNA levels in normal HMEC cells in culture.
2. By using the MatInspector V2.2 computer program, we identified two conserved half-sites separated by four nucleotides (DR-4), as well as a DR-5 RARE in the 5' flanking region of the human LRAT promoter region.
3. We isolated the 2.3-kb 5'-flanking promoter region of the human LRAT gene. The luciferase activity of this promoter region in MDA-MB-231 cells was one sixth of that in HMEC cells at 48 h, which reflects the difference in LRAT mRNA levels between the two cell lines.
4. The striking difference in the promoter activity of this 2.3-kb 5'-flanking region between the HMEC cells and MDA-MB-231 cells is important. It suggests that this region harbors *cis*-elements which are involved in the loss of LRAT expression in human breast cancer cells.
5. The 2.3 kb promoter sequence does not harbor any *cis* element(s) required for RA or ROL responsiveness in HMEC and MDA-MB-231 cells.
6. RA treatment increases LRAT mRNA levels in F9 Wt cells at late times (72 h). There was no apparent difference in the LRAT mRNA levels between the F9 Wt cells and F9 RARγ-/- cells, indicating that RARγ is not involved in the regulation of LRAT gene expression. This supports the concept that RARα is the predominant RAR isotype mediating RA- induced LRAT gene expression.

Reportable Outcomes

Differences in Global Gene Expression Between Wild Type and RAR gamma Knockout F9 Teratocarcinoma Stem Cells after Retinoic Acid Treatment Dan Su and Lorraine J Gudas (manuscript in preparation)

Conclusions

In order to understand the molecular mechanisms that mediate LRAT transcription by RA in normal human mammary epithelial cells versus breast carcinoma cells, we isolated and characterized the promoter region of the human *LRAT* gene and tested its activity in normal mammary epithelial cells versus human breast carcinoma cells. Meanwhile, in order to determine if RARγ is involved in the regulation of LRAT gene expression, we tested LRAT mRNA levels in Wt and RARγ -/- F9 cells, an epithelial type of cell, which can synthesize retinyl esters. The RT-PCR analysis indicated that LRAT expression was lost in the MDA-MB-231 breast cancer cells. Surprisingly, we did not observe RA associated increase in LRAT mRNA levels in HMEC cell line in culture. By using the MatInspector V2.2 computer program, we identified two conserved half-sites separated by four nucleotides (DR-4), as well as a DR-5 RARE in the 5' flanking region of the LRAT promoter region. We isolated the 2.3-kb 5'-flanking region of the human LRAT gene. The luciferase activity of this promoter region in MDA-MB-231 cells was one sixth of that in HMEC cells at 48 h, suggesting that this region harbors cis-elements contributing to the loss of LRAT expression in human breast cancer cells. RA treatment increases LRAT mRNA levels in F9 Wt cells at late times (72 h). There was no apparent difference in the LRAT mRNA levels detected between the F9 Wt cells and F9 RARγ-/- cells, indicating that RARγ is not involved in the transcriptional regulation of the human LRAT gene.

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